Remarks

Claims 25-41 were the subject of the office action dated April 20, 2006. Claims 27, 29, and 31-32 are canceled without prejudice as explained below. Claims 42-51 are added by this Response. Thus, claims 25-26, 28, 30, and 33-51 are now before the Examiner.

Claim 27, relating to potentiators, is combined into claim 25, and examples of toxin complex toxin proteins are now specified in claim 25. Claim 27 is canceled without prejudice. This should render moot the objection to claim 27 and the rejection of claim 27 for indefiniteness. The applicants wish to note, however, that the specification clearly explains the term "toxin complex protein toxin" in light of the art. *See e.g.* paragraphs 35-43 and 134-141. That is, certain toxin complex proteins were known to have stand-alone insecticidal activity, and some toxin complex proteins were known to enhance or potentiate the activity of the stand-alone protein toxins. The meaning of the term "toxin complex protein toxin" would thus be clear to one skilled in the art. In addition, Waterfield *et al., TRENDS in Microbiology*, Vol. 9, No. 4, (April 2001), pp. 185-191 , "The *tc* genes of *Photorhabdus*, a growing family," copy attached, shows that the term "*tc*" for toxin complex genes of *Photorhabdus* and *Xenorhabdus* is a term of the art. *See also* page 186 and Figure 1 of that reference.

Claim 41 is amended (without prejudice) to match the insects tested in Example 10.

Claims 25-41 stand rejected as lacking an adequate written description. The applicants respectfully traverse this rejection. The specification clearly shows that TcaC- and TccC-type potentiators were obtained from two different strains of *Paenibacillus*. (The specification also shows that TcaA and TcaB toxin complex proteins were obtained from these strains.) Thus, one skilled in the art would not question whether the applicants possessed the invention as claimed. In light of the foregoing, the withdrawal of this rejection is respectfully requested.

Claims 25-41 stand rejected under 35 USC §102(b) as being anticipated by WO 94/21795 (Warren). The applicants respectfully traverse this rejection. Warren does not teach "toxin complex" proteins as described by Waterfield *et al.* and as described in detail in the subject application. Thus, the methods now claimed could not have been anticipated by Warren. In light of the foregoing, the withdrawal of this rejection is respectfully requested.

Claims 25-41 stand rejected under 35 USC §103(a) as being obvious in light of WO 94/21795 (Warren). The applicants respectfully traverse this rejection. Warren does not teach "toxin complex" proteins as described by Waterfield *et al.* and as described in detail in the subject application. Thus, the methods now claimed could not have been obvious in light of Warren. In light of the foregoing, the withdrawal of this rejection is respectfully requested.

The applicants believe that this application is in condition for allowance, and such action is earnestly solicited.

The Assistant Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 and 1.17 as required by this paper to Deposit Account 19-0065.

The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

Jay M. Sanders
Patent Attorney
Registration No. 39,355
Phone No.: 352-375-8100
Fax No.: 352-372-5800
Address: P.O. Box 142950
Gainesville, FL 32614-2950

JMS/ehm
Attachments: Waterfield *et al.*, April 2001
Petition and Fee for Extension of Time

References

1 Achtman, M. (1996) A surfeit of YATMs? *J. Clin. Microbiol.* 34, 1870
2 Musser, J.M. (1996) Molecular population genetic analysis of emerged bacterial pathogens: selected insights. *Emerg. Infect. Dis.* 2, 1–17
3 Spratt, B.G. *et al.* (1999) Bacterial population genetics, evolution and epidemiology. *Proc. R. Soc. London B Biol. Sci.* 354, 701–710
4 Ochman, H. *et al.* (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299–304
5 Reid, S.D. *et al.* (2000) Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 406, 64–67
6 Feil, E.J. *et al.* (2001) Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proc. Natl. Acad. Sci. U. S. A.* 98, 182–187
7 Gupta, S. *et al.* (1996) The maintenance of strain structure in populations of recombining infectious agents. *Nat. Med.* 2, 437–442
8 Gupta, S. *et al.* (1999) The effects of host heterogeneity on pathogen population structure. *Philos. Trans. R. Soc. London B Biol. Sci.* 354, 711–719
9 Maiden, M.C.J. (2000) High-throughput sequencing in the population analysis of bacterial pathogens. *Int. J. Med. Microbiol.* 290, 183–190
10 Popoff, M.Y. *et al.* (1998) Supplement 1997 (no. 41) to the Kauffmann-White scheme. *Res. Microbiol.* 149, 601–604
11 Selander, R.K. *et al.* (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* 51, 837–884
12 Maiden, M.C.J. *et al.* (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* 95, 3140–3145
13 Suerbaum, S. *et al.* (1998) Free recombination within *Helicobacter pylori*. *Proc. Natl. Acad. Sci. U. S. A.* 95, 12619–12624
14 Achtman, M. *et al.* (1999) *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* 96, 14043–14048
15 Feil, E.J. *et al.* (1999) The relative contribution of recombination and mutation to the divergence of clones of *Neisseria meningitidis*. *Mol. Biol. Evol.* 16, 1496–1502
16 Holmes, E.C. *et al.* (1999) The influence of recombination on the population structure and evolution of the human pathogen *Neisseria meningitidis*. *Mol. Biol. Evol.* 16, 741–749
17 Selander, R.K. *et al.* (1980) Genetic diversity and structure in *Escherichia coli* populations. *Science* 210, 545–547
18 Maynard Smith, J. *et al.* (1993) How clonal are bacteria? *Proc. Natl. Acad. Sci. U. S. A.* 90, 4384–4388
19 Feavers, I.M. *et al.* (1996) Antigenic diversity of meningococcal outer membrane protein PorA has implications for epidemiological analysis and vaccine design. *Clin. Diagn. Lab. Immunol.* 3, 444–450
20 Peeters, C.C. *et al.* (1999) Immunogenicity of various presentation forms of PorA outer membrane protein of *Neisseria meningitidis* in mice. *Vaccine* 17, 2702–2712
21 Wiertz, E.J. *et al.* (1991) T cell recognition of *Neisseria meningitidis* class 1 outer membrane proteins. Identification of T cell epitopes with selected synthetic peptides and determination of HLA restriction elements. *J. Immunol.* 147, 2012–2018
22 Suker, J. *et al.* (1994) The *porA* gene in serogroup A meningococci: evolutionary stability and mechanism of genetic variation. *Mol. Microbiol.* 12, 253–265
23 Artenstein, M.S. *et al.* (1971) Serologic studies of meningococcal infection and polysaccharide vaccination. *J. Infect. Dis.* 124, 277–288
24 Caugant, D.A. *et al.* (1987) Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. *J. Bacteriol.* 169, 2781–2792
25 Caugant, D.A. *et al.* (1990) Clones of serogroup B *Neisseria meningitidis* causing systemic disease in the Netherlands, 1958–1986. *J. Infect. Dis.* 162, 867–874
26 Anthony, B.F. *et al.* (1976) The dynamics of streptococcal infections in a defined population of children: serotypes associated with skin and respiratory infections. *Am. J. Epidemiol.* 104, 652–666
27 Bessen, D.E. *et al.* (1996) Genetic correlates of throat and skin isolates of group A streptococci. *J. Infect. Dis.* 173, 896–900
28 Rakonjac, J.V. *et al.* (1995) DNA sequence of the serum opacity factor of group A streptococci: identification of a fibronectin-binding repeat domain. *Infect. Immun.* 63, 622–631
29 Areschoug, T. *et al.* (1999) Group B streptococcal surface proteins as targets for protective antibodies: identification of two novel proteins in strains of serotype V. *Infect. Immun.* 67, 6350–6357
30 Maiden, M.C.J. *et al.* (1999) Meningococcal conjugate vaccines: new opportunities and new challenges. *Lancet* 354, 615–616
31 Spratt, B.G. *et al.* (2000) Prevention of pneumococcal disease by vaccination: does serotype replacement matter? *Lancet* 356, 1210–1211

The *tc* genes of *Photorhabdus*: a growing family

Nicholas R. Waterfield, David J. Bowen, Jacqueline D. Fetherston, Robert D. Perry and Richard H. ffrench-Constant

The toxin complex (*tc*) genes of *Photorhabdus* encode insecticidal, high molecular weight Tc toxins. These toxins have been suggested as useful alternatives to those derived from *Bacillus thuringiensis* for expression in insect-resistant transgenic plants. Although *Photorhabdus luminescens* is symbiotic with nematodes that kill insects, *tc* genes have recently been described from other insect-associated bacteria such as *Serratia entomophila*, an insect pathogen, and *Yersinia pestis*, the causative agent of bubonic plague, which has a flea vector. Here, recent advances in our understanding of the *tc* gene family are reviewed in view of their potential development as insect-control agents.

Following the widespread deployment of insect-resistant transgenic plants expressing δ-endotoxins from the bacterium *Bacillus thuringiensis* (Bt), there are growing concerns about the development of insect resistance to Bt (Refs 1–3) and the search for alternative protein toxins is ongoing. As part of this search, we and others have cloned the toxin complex (*tc*) genes from *Photorhabdus luminescens*[4,5]. These genes encode high molecular weight, insecticidal toxin complexes, or Tcs, with oral insecticidal activities comparable with those of Bt toxins[4]. *P. luminescens* is a bacterium symbiotic with round worms that invade and kill insects (entomopathogenic nematodes). Following invasion of an insect by the nematode, the bacteria are released from the nematode gut into the open circulatory system (hemocoel) of the insect[6]. Here, they are thought to release a variety of virulence factors, including the Tc toxins, which aid in the killing and bioconversion of the insect host[7]. In *P. luminescens*,

http://tim.trends.com 0966-842X/01/$ – see front matter PII: S0966-842X(01)01978-3

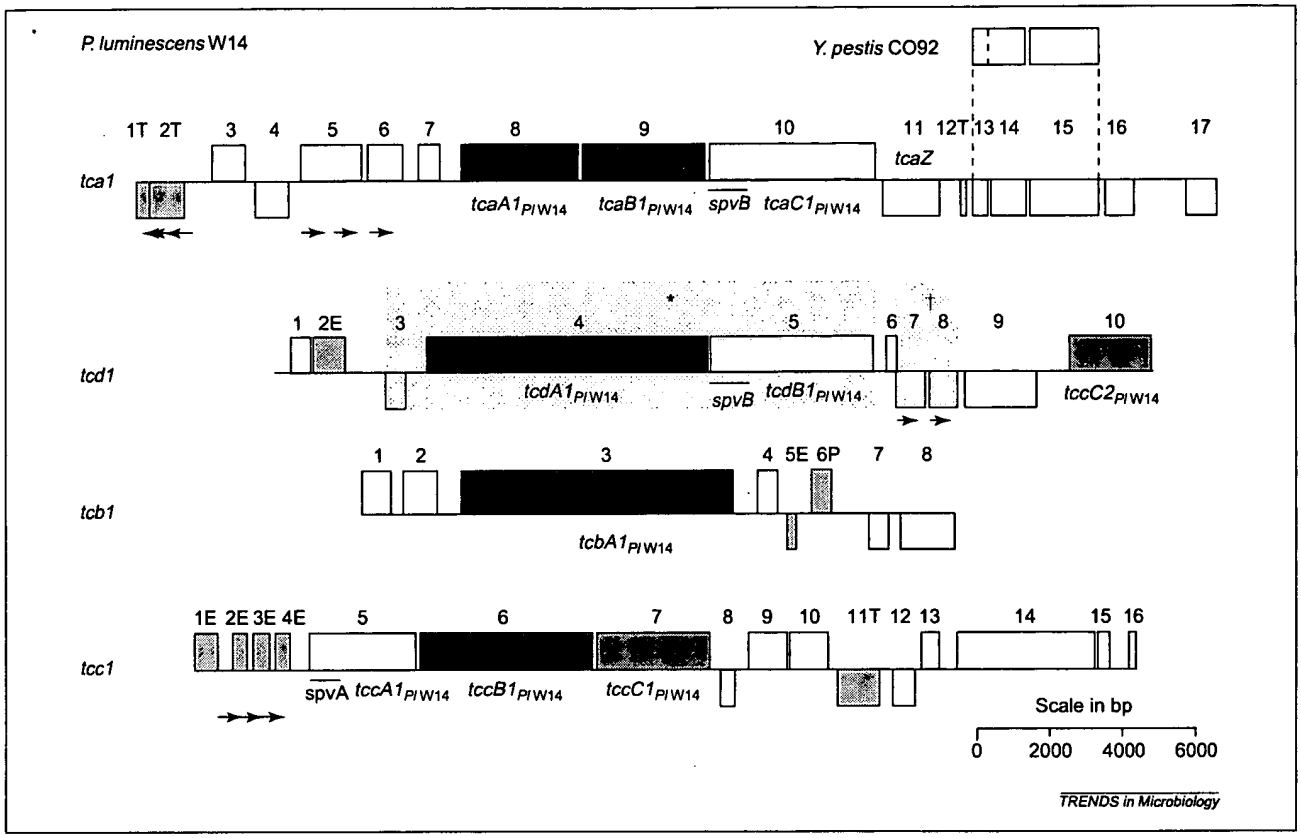

Fig. 1. Genomic organization of the original four *tc* loci from the nematode symbiont *Photorhabdus luminescens* (Pl) W14. The genes encoding the three conserved elements are color coded: TcaAB-like or Tcb/TcdA-like, red; TcaC-like, yellow and TccC-like, green. Note the presence of extrachromosomal elements [dark gray open reading frames (ORFs)] surrounding the *tc* loci (P, phage-derived; T, transposon-associated; E, sequences such as colicins that are normally of extrachromosomal origin). Also note the presence of ORFs that are directly repeated (arrows). The top BLASTX matches for those ORFs returning a significant match are given in Table 1. Further descriptions of these sequences are given in GenBank accession numbers AF346497–AF346500. Shaded regions are highly similar at the nucleotide level between different *Photorhabdus* strains (see also the shaded regions marked * and † in Fig. 2).

Nicholas R. Waterfield
Richard H. ffrench-Constant*
Dept of Biology and Biochemistry, University of Bath, Bath, UK BA2 7AY.
*e-mail: bssrfc@bath.ac.uk

David J. Bowen
Dept of Entomology, University of Wisconsin-Madison, Madison, WI 53706, USA.

Robert D. Perry
Jacqueline D. Fetherston
Dept of Microbiology and Immunology, University of Kentucky College of Medicine, Lexington KT 40536, USA.

the *tc* genes appear to be encoded alongside genes for other putative virulence factors. Most recently, homologs of the *tc* loci have been identified not only in other bacteria symbiotic with nematodes, such as *Xenorhabdus nematophilus*, but also in bacteria that use insects either as hosts (*Serratia entomophila*) or as vectors (*Yersinia pestis*). This raises questions not only about the role of these toxins in the different life cycles of these bacteria but also about their mode of action. Here, we review what we know about these new insecticidal toxins and describe the commonalities between the loci encoding them in different bacteria.

The *tc* loci of *Photorhabdus*

Toxin purification and gene cloning

The Tc toxins were first purified from *P. luminescens* strain W14 (Refs 5,8). Four toxin complexes, termed Tca, Tcb, Tcc and Tcd, were separated by column- and high-performance liquid chromatography[4]. Each of these complexes migrates as a single species on a native gel but resolves into numerous polypeptides on a denaturing polyacrylamide gel. Addition of all the different polypeptide components suggests that each complex might have a molecular weight of over one million[4]; however, the precise composition of each native complex and its subunit stochiometry remains obscure. The four loci that encode each of these complexes, termed *tca*, *tcb*, *tcc* and *tcd*, were cloned using antibodies raised against a mixture of all four complexes. At the time, these loci showed little homology to any sequences in GenBank and had no consensus secretion signals[4]. The only limited similarity was to *Salmonella* plasmid-borne virulence factor (Spv) proteins. Thus, the amino termini of TcaC, TcdB and TccA each show similarity to either SpvA or SpvB (Fig. 1). In *Salmonella*, SpvB enhances the survival of the bacteria in macrophages[9]. Recently, the carboxyl terminus of SpvB was shown to be an ADP-ribosyltransferase[10] but the function of the amino terminus, which shows similarity to TcaC (Ref. 10), is unknown. It is likely that the Tc toxins, like the Spv proteins, are fusion proteins of several different moieties of differing functions.

The predicted Tc proteins have three areas of striking similarity with each other[4]. Thus, despite the apparent complexity of the loci, only three basic types of genetic element are apparent: (1) *tcaAB* or *tcdA*-like genes; (2) *tcaC* or *tcdB*-like genes; and (c) *tccC*-like genes. Homologs of these same three genetic

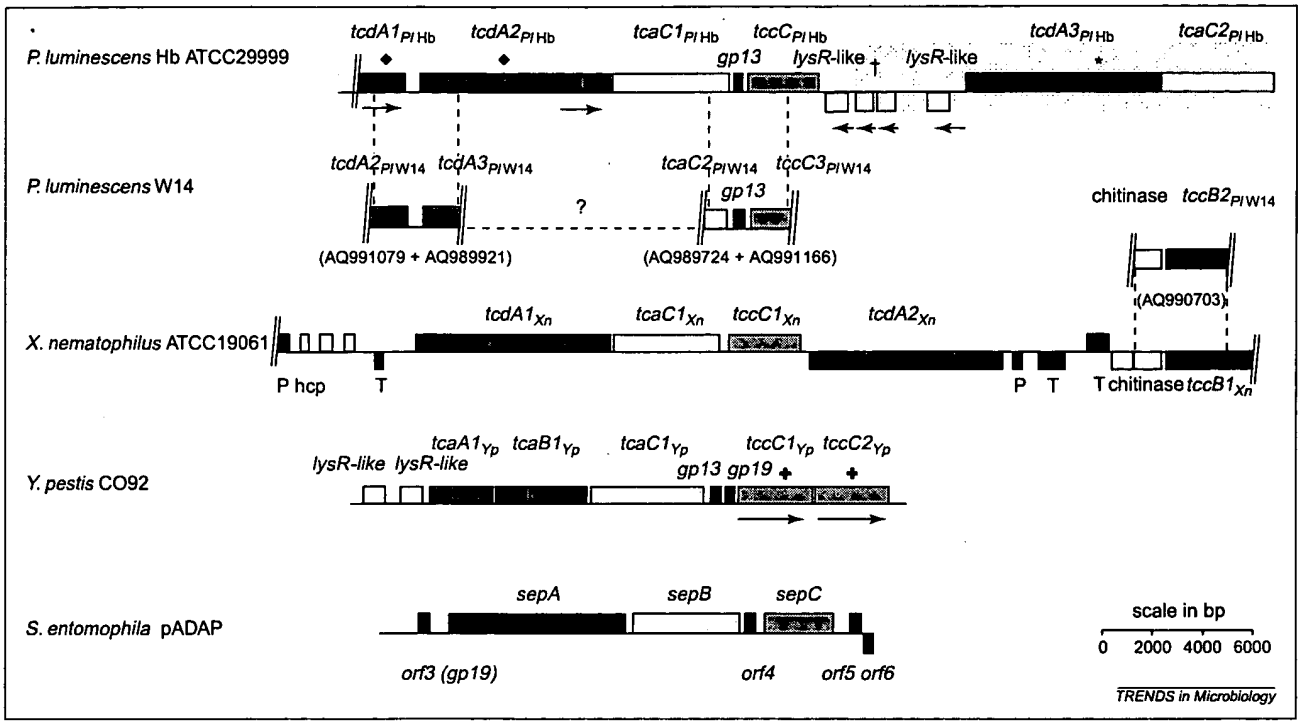

Fig. 2. Genomic organization of *tc*-like genes from nematode symbiotic [*Photorhabdus luminescens* (*Pl*) and *Xenorhabdus nematophilus* (*Xu*)] and non-symbiotic (*Yersinia pestis* and *Serratia entomophila*) bacteria. The genes encoding the three conserved toxin elements (TcaAB-like, TcaC-like and TccC-like) are colour coded (red, yellow and green, respectively). These similar loci constitute a conserved block consisting of one or two toxin open reading frames (ORFs), phage-related sequences and then a *tccC*-like locus. Note also the presence of elements putatively of extrachromosomal origin, specifically those associated with phage genes *gp13* and *gp19* and directly duplicated sequences (arrows under the ORFs). The symbols * and ◆ indicate ORFs highlighted in the phylogenetic trees in Fig. 3. The symbols * and † indicate ORFs highlighted in Fig. 1. The symbol ✚ indicates tandem gene duplications (see Fig. 4). Shaded regions are highly similar at the nucleotide level between different *Photorhabdus* and *Xenorhabdus* strains (see also Fig. 1). Nucleotide sequences for *P. luminescens* Hb ATCC29999 and *X. nematophilus* ATCC 19061 are from the patents of Kramer *et al.* 1999, World Intellectual Property, 99/42589 and Jarrett *et al.* 1997, World Intellectual Property, 97/02284 respectively. Nucleotide sequences from *P. luminescens* W14 are from a sample sequence and accession numbers are shown below the diagram.

elements have been found in several other bacteria and their conservation suggests that these three basic components are all necessary for oral activity of the toxin complexes against insects.

Putative virulence genes surround the *tc* loci

Extended sequencing around the *tc* loci has revealed 40 putative new open reading frames (ORFs) in the W14 genome (Fig. 1), of which 15 return no significant match from a BLASTX search of the existing GenBank databases. This suggests that the *tc* genes could be surrounded by other rapidly evolving genes potentially involved in virulence. Several of these genes show clear matches with potential pathogenicity and toxic factors in other bacteria (Table 1). Notable amongst these are two ORFs upstream of *tca* (5 and 6 in Fig. 1), whose predicted amino acid sequences show similarity to the vertebrate heme-scavenging molecule hemopexin and which we have termed photopexin A and B. We have modeled the predicted amino acid sequence of photopexin onto the crystal structure of hemopexin and shown a surprisingly good fit[11]. This raises the possibility that *Photorhabdus* photopexin acts as an iron-scavenging molecule in the insect host where iron concentrations are likely to be very low[11]. Upstream of the *tcc* locus are several ORFs with similarity to colicins and their associated immunity proteins. Colicins and their immunity proteins are toxin–anti-toxin systems used to kill non-self bacteria in bacterial competition[12]. This suggests that different *Photorhabdus* strains might carry different colicins to kill other strains of bacteria that invade the insect host, including other strains of *Photorhabdus*. Alongside the genes encoding photopexin A and B and colicin-like factors, several flanking ORFs also have high levels of similarity to transposases and phage-related proteins, providing a potential mechanism of transfer for these loci between strains or even species. Several regions adjacent to the *tca*, *tcc* and *tcd* loci also show clear duplications (arrows in Fig. 1), again suggesting possible recent DNA rearrangements.

Homologs in a growing number of bacteria

Homologs in other nematode symbionts

Since the discovery of *tc* genes in *P. luminescens* strain W14, orally active cosmid clones have also been recovered from a different strain of *P. luminescens*, strain Hb, and also from a different bacterium, *X. nematophilus*, which is symbiotic with a separate group of nematodes (Fig. 2). Notably, both these large (~40 kb) orally toxic *Escherichia coli* clones contain genes encoding all three conserved toxin elements: TcaAB-like or Tcb/TcdA-like elements, TcaC-like

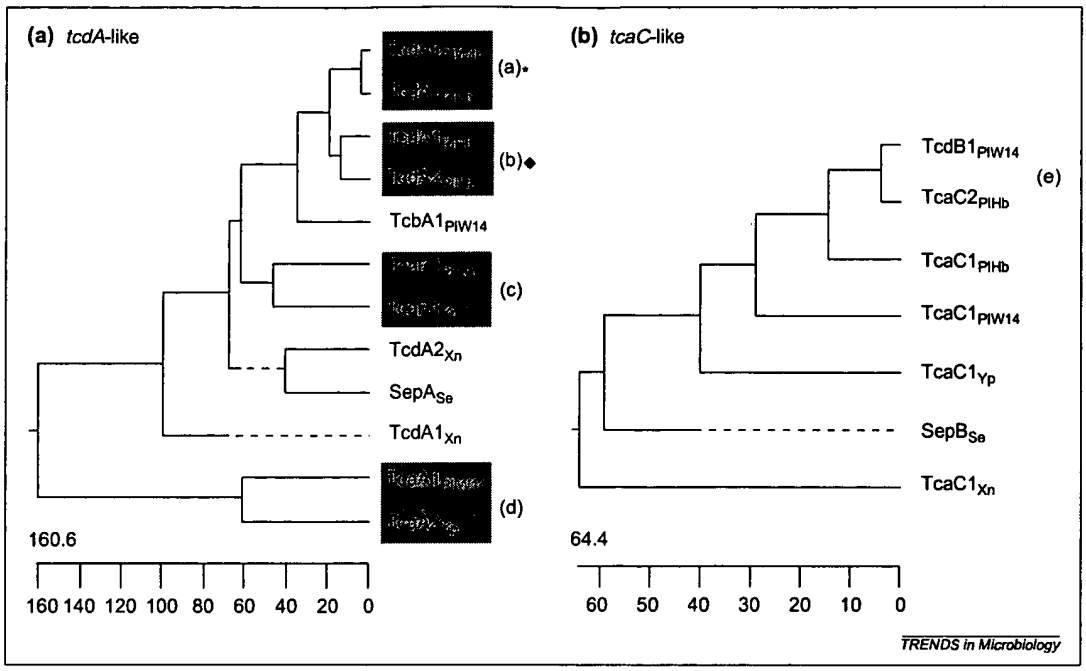

Fig. 3. Phylogeny of (a) *tcdA*-like and (b) *tcaC*-like genes. Note that some *tcdA*-like open reading frames (ORFs) (•) are highly related at the nucleotide sequence level, despite the fact that they come from different *Photorhabdus luminescens* strains. The *tcdA*-like sequences $tcaB1_{PIW14}$ and $tcaB1_{Yp}$ have their closest relatives in different species (*Photorhabdus* and *Yersinia pestis*, respectively). Note also that other *tcdA*-like sequences are almost identical ($tcdA1_{Hb}$ and $tcdA2_{Hb}$, ◆) and probably reflect direct gene duplications. Clades of the phenogram are labelled 'a–e' in reference to discussion in the text. The phylogeny (Clustal algorithm) is a phenogram constrained to have balanced branches and therefore the distance lengths shown in the scale reflect only approximate estimates of divergence from the ancestral node.

elements and a TccC-like element. Interestingly, elements of the genomic organization of the loci from *P. luminescens* Hb and *X. nematophilus* are conserved (Fig. 2). New *tc*-like loci have been discovered in *P. luminescens* W14 via a random genomic sample sequence[13], confirming not only that there are more *tc*-like loci in *P. luminescens* W14 than the original four but also that these could have further homologs in other *Photorhabdus* and *Xenorhabdus* strains. Further genomic sequencing in a range of different strains will help to resolve how many *tc* loci there are per strain, and how conserved they are between different strains and species.

Table 1. Putative functions of ORFs found around the *tc* loci of *Photorhabdus luminescens* W14[a]

ORF	BLASTX match	Score	Organism
***tca* locus**			
1	Transposase, YPMT1.71	3e-80	*Yersinia pestis*
2	Transposase, YPMT1.71	2e-74	*Y. pestis*
5	Limunectin (Photopexin A)	3e-12	*Limulus*
6	Limunectin (Photopexin B)	1e-7	*Limulus*
12	Transposase, IS630	1e-30	*Salmonella dublin* pSDL2
13	Hypothetical protein	6e-61	*Y. pestis*
14	Hypothetical protein	e-134	*Y. pestis*
15	Hypothetical protein	e-148	*Y. pestis*
***tcb* locus**			
5	Adherence factor plasmid	3e-04	*Escherichia coli* pB171
6	Phage head assembly protein	0.31	Bacteriophage D29
7	Vacuolating cytotoxin	2.2	*Helicobacter pylori*
***tcc* locus**			
1	Colicin E4	7e-37	*E. coli* pColE4
2	Colicin E4 immunity protein	2e-23	*E. coli* pColE4
3	Pyocin S3 immunity protein	8e-05	*Pseudomonas aeruginosa*
4	Pyocin S3 immunity protein	3e-07	*P. aeruginosa*
9	toxic cation resistance, YaaN	8e-66	*Bacillus subtilis*
11	Transposase	e-178	*E. coli* pCollb-P9
14	Butirosin-biosynthetic, BrtA	5e-50	*Bacillus circulans*
***tcd* locus**			
2	Phage abortive infection protein	6.9	*Lactococcus lactis*
9	Hypothetical protein, VC1418	9e-15	*Vibrio cholerae*

[a]Abbreviations: ORF, open reading frame; *tc*, toxin complex.

Comparisons of the different loci between species also highlight the frequent occurrence of phage-related sequences between *tc*-like sequences, notably those similar to bacteriophage genes *gp13* and *gp19*. In *Y. pestis* CO92 (Fig. 2), these phage-related proteins correspond to a holin/porin and a lysozyme-like gene from the *Salmonella* phage PS3. The presence of these phage-like genes raises the intriguing possibility that the *tc* genes might in fact be part of a cryptic prophage, in an analogous fashion to *nucC* of *Serratia marcescens*[14]. If this hypothesis can be substantiated, it suggests that the Tc toxins are released following cell lysis mediated by these phage-like genes, and that Tc export is therefore independent of classical secretion mechanisms. This would help explain how such large protein complexes can readily escape from the bacterial cell.

Homologs in non-symbiotic bacteria

Most recently, homologs of *tc* genes have also been found in two other insect-associated bacteria, *S. entomophila*[15] and *Y. pestis* (Fig. 2). In *S. entomophila*, the *tc*-like genes *sepA*, *sepB* and *sepC* are sufficient to cause amber disease in the beetle grass grub *Costelytra zealandica*[15]. This reaffirms the assumption, derived from the comparisons of nematode symbiotic bacteria discussed above, that all three of these conserved

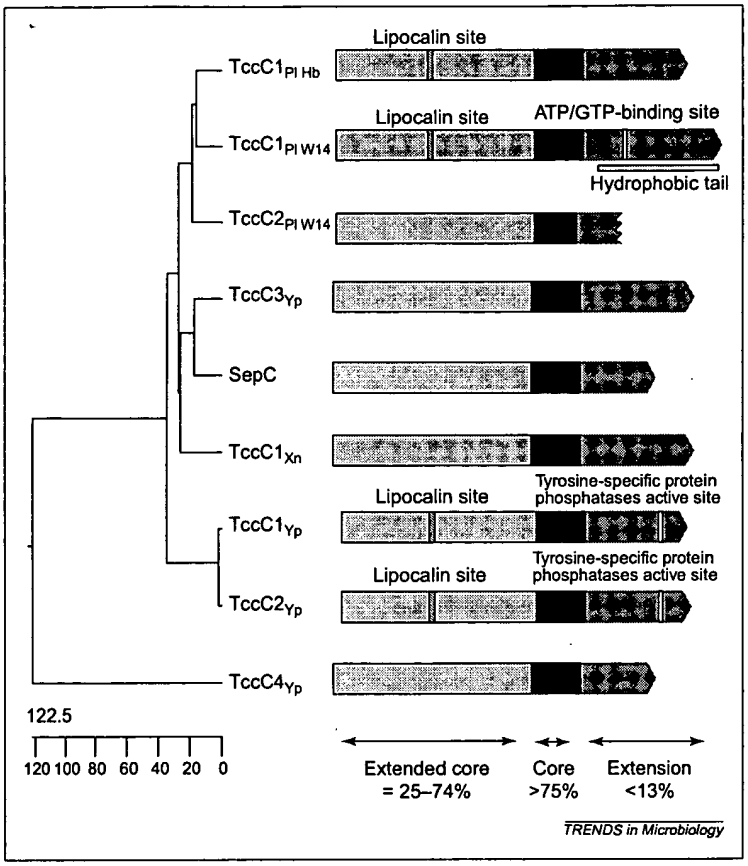

Fig. 4. Phylogeny of the predicted TccC-like proteins (based only on their conserved carboxy-terminal halves) and a schematic representation of their core-extension-like structure. The 'core' represents the most highly conserved domain (>75% predicted amino acid identity to the equivalent region in $tccC1_{PIW14}$). The 'extended core' is a region of intermediate identity (25–74%) and the 'extensions' essentially comprise a series of unrelated domains (<13% identity). Note again that (as in copies of *tcdA* in Fig. 3), *tccC*-like sequences that are almost identical ($tccC1_{Yp}$ and $tccC2_{Yp}$) appear to be tandem gene duplications (see ✚ in Fig. 2). The phylogeny (Clustal algorithm) is a phenogram constrained to have balanced branches and therefore the distance lengths shown in the scale reflect only approximate estimates of divergence from the ancestral node.

elements (in this case *sepA*, *sepB* and *sepC*) are necessary and sufficient for oral toxicity. In *S. entomophila*, these three *tc*-like genes are located on a 115-kb plasmid, pADAP, which can confer on *E. coli* the ability to cause amber disease[15]. At this stage, the discovery of plasmid-borne *tc*-like genes in *Serratia* appears to be unique. However, it is a formal possibility that *tc* genes could be transmitted between species on plasmids and that they could also be plasmid-associated at times in other bacteria (including *Photorhabdus*).

Relationships between *tc*-like genes

Phylogenies of tc*-like genes between species*

To begin to understand how the *tc* homologs in these different bacterial species are related to one another, we have carried out phylogenetic analyses on all the TcaC-like and TcdA-like predicted protein sequences. These show clear suggestions of recent horizontal transfer. For example, the TcaA-like sequences in clade 'd' and the TcaB-like sequences in clade 'c' (Fig. 3a) are the most closely related and yet they come from very different bacteria, namely *P. luminescens* and *Y. pestis*. Similar examples can be found within *tcaC*-like genes of clade 'e' (Fig. 3b), where the most closely related sequences come from two different *Photorhabdus* strains. However, the mechanisms whereby such potential horizontal transfers take place and their relationship to the plasmid, transposon or phage-related sequences surrounding the *tc* loci remain to be established.

Questions for future research

- What is the subunit composition of native Tc complexes?
- How are the Tc toxins exported from the bacterial cell?
- How do the Tc toxins interact with the insect gut?
- What is the mode of action of Tc toxins?
- What is their toxicity to vertebrates?
- What is the role of Tc toxins in *Yersinia pestis*?
- How are *tc* genes transferred between different species?
- How many other species of bacteria contain *tc* genes?

The tccC*-like family: a novel set of toxins?*

As discussed earlier, three Tc elements are necessary for oral toxicity: a Tcb/TcdA-like or TcaAB-like element, a TcaC-like element and a TccC-like element. In the case of Tcd, expression of TcdA and TcdB (TcaC-like) together in *E. coli* is sufficient to produce toxin complexes clearly visible by transmission electron microscopy (TEM; R.H. ffrench-Constant *et al.*, unpublished). However, these TcdAB-containing complexes are not themselves orally toxic to insects. For oral toxicity, co-expression of TccC is necessary. Addition of TccC does not change the physical appearance of the complexes under TEM but it is essential to render them orally toxic. The simplest but not the only hypothesis to explain these data is that TcdAB encodes a delivery system and TccC itself is the active delivered toxin.

Comparison of the predicted amino acid sequences of all the known TccC-like proteins shows that they form part of a family similar to core-extension proteins. Thus they have a highly conserved 'core', a less conserved 'extended core' and a highly variable 'extension' and 'leader' (Fig. 4). This organization is very similar to that of the *rhs* genes of *E. coli*, which are thought to be an ancient gene family but are of unknown function[16]. The extensions of the TccC-like proteins are not only very variable but some also carry consensus sites for either ATP or GTP binding or tyrosine-specific protein-phosphatase activity (Fig. 4), supporting the hypothesis that they are toxins. This suggests that

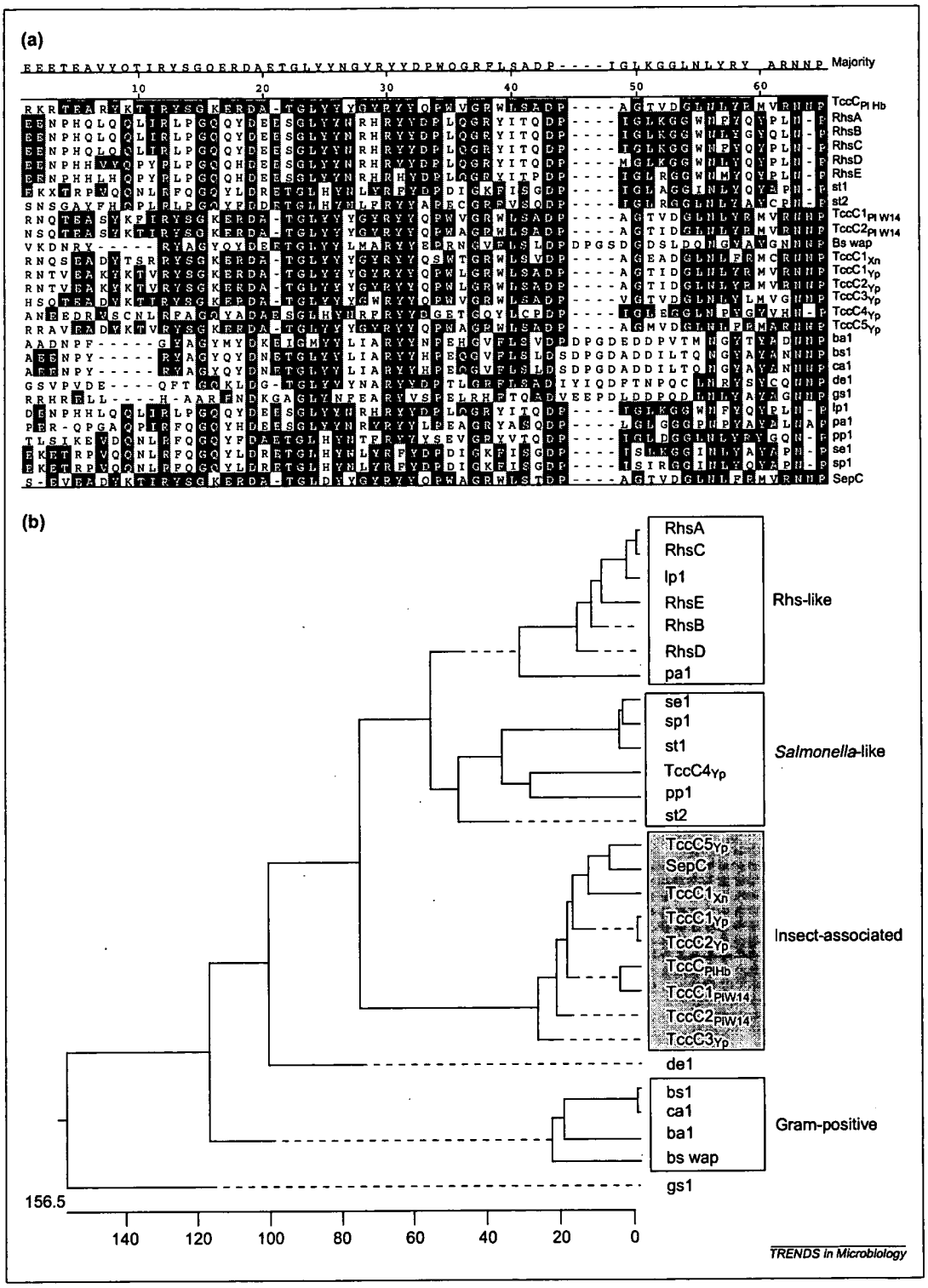

Fig. 5. Alignment and phylogeny of Rhs/TccC core-like sequences from both Gram-negative and Gram-positive bacteria. (a) The alignment corresponds to the region containing the 'core' of TccC-like sequences (Fig. 4), which ends with a conserved proline. A majority consensus sequence is given above. (b) A phylogeny of the same core-like sequences. Note that four broad clades are determined: an Rhs-like group, a *Salmonella*-like group, an insect-associated group (with representatives from both the nematode symbionts *Photorhabdus* and *Xenorhabdus* as well as from *Yersinia pestis*) and a clade containing only Gram-positive bacteria. Aside from the TccC-like sequences discussed above, the remaining sequences were obtained by BLAST searching against the following unfinished bacterial genomes (http://www.ncbi.nim.gov/Microb_blast/unfinishedgenome.html): ba1, *Bacillus anthracis*; bs1, *Bacillus stearothermophilus*; bs wap, *Bacillus subtilis* wall-associated protein (GenBank accession no. D29985); ca1, *Clostridium acetobutylicum*; de1, *Dehalococcoides ethenogenes*; gs1; *Geobacter sulfurreducens*; lp1, *Legionella pneumophila*; pa1, *Pseudomonas aeruginosa*; pp1, *Pseudomonas putida*; SepC, *Serratia entomophila*; se1, *Salmonella enteritidis*; sp1, *Salmonella paratyphi* A; st1 and st2, *Salmonella typhimurium* LT2. We acknowledge and thank the Sanger Centre, TIGR and UW Genome Project for access to their data.

the different extensions might enable the different TccC homologs to modulate host cells in alternative virulence functions.

Alignment of the core of the *tccC*-like genes described a highly conserved consensus amino acid sequence (Fig. 5a); searching of the current databases with this consensus revealed that numerous other *tccC*-like genes are predicted in other bacteria. Phylogenetic analysis of their amino acid sequences shows four distinct families (Fig. 5b): (1) an insect-

associated family containing sequences from bacteria either infecting insects or using them as vectors; (2) an Rhs-like clade, typified by the Rhs elements of *E. coli*; (3) a clade of sequences typified by homologs from *Salmonella*. These are most closely related to the Rhs-like elements but are clearly distinct; and (4) a clade representing homologs within Gram-positive bacteria. Thus, the *tccC*/*rhs*-like family is broad and sufficiently ancient to possess progenitors that might have been present before the split of Gram-positive and Gram-negative bacteria. If the functionality of the different putative active sites found in the TccC homologs can be proven, this could confirm that the TccC proteins are a broadly distributed and novel class of delivered toxins.

Conclusions and future directions

Many questions remain to be answered about the growing *tc*-like gene family. Furthermore, as interest increases in these genes for deployment in insect-resistant transgenic plants, some of these questions are becoming increasingly important. Most notable perhaps is the mode of action of the Tc toxins themselves. Examination of the histopathology of insects fed with purified Tca shows that effects are specific to the insect midgut whose treated epithelium blebs into the midgut lumen and eventually disintegrates[17]. Interestingly, when Tca is injected directly into the insect hemocoel (a mode of action perhaps more similar to its normal delivery from the bacteria in the hemocoel), the cells of the midgut epithelium are again affected. However, this time, they round up and appear to detach from the basement membrane[17]. These observations obviously raise more questions than they answer. For example, how can the Tc toxins interact with both sides of the insect gut and which specific cellular processes are they interfering with? Aside from the similarities of areas of the Tc proteins to the *Salmonella* Spv proteins, the Tc toxins also carry a conserved RGD motif[15]. The RGD motif is found in cell-surface adhesins produced by the human pathogen *Bordetella pertussis* and is implicated in enhancing bacterial binding to eukaryotic cells[18,19]. This motif has therefore been suggested to play a role in the attachment of Tc toxins and/or the bacteria to insect cells[15]. Again, these observations raise specific questions about the mode of action of these novel toxins.

Finally, the discovery of the *tc* genes in such a range of bacteria also raises the question of their biological role in different species. In *P. luminescens*, and probably in *S. entomophila*, the Tc toxins are used to destroy the midgut of the insect host[17]. This would render the infected host incapable of further feeding. However, the role of such genes in *Y. pestis* is not so clear. In this species, the bacterium is spread from host to host via the blood-feeding activities of fleas. Fleas infected with *Y. pestis* have blocked midguts, which is thought to facilitate regurgitation of the infected blood meal into new hosts on further feeding[20]. Midgut blockage has been associated with the *hms* genes[21] but perhaps the *tc* genes are also required alongside *hms* genes for blockage or perhaps they are necessary for survival of *Y. pestis* in the flea midgut itself. Whatever their role in different species, it seems likely that further *tc* gene homologs will be found in the wide variety of bacteria that infect invertebrates and that they probably represent a large gene family widely distributed in the Enterobacteriaceae.

Acknowledgements
This research was supported by grants from the BBSRC and the Wellcome Trust (J.I.F.) to R.ff-C. J.D.F. and R.D.P. are supported by Public Health Service Grants AI25098, AI33481 and AI42738 from NIH. We thank Thomas Rocheleau and Cathy Grutzmacher for nucleotide sequencing of *P. luminescens* W14 DNA. The *Y. pestis* CO92 sequence data were produced by the *Y. pestis* sequencing group at the Sanger Centre (ftp://ftp.sanger.ac.uk/pub/pathogens/yp/YP.dbs). The *Y. pestis* K1M10+ sequence data were produced by the UW Genome Project (http://magpie.genome.eisc.edu/cgi-bin/Authenticate.gi). For data collections, both sites were last accessed by the authors on 1 September 2000.

References

1 McGaughey, W.H. (1985) Insect resistance to biological insecticide *Bacillus thuringiensis*. *Science* 229, 193–195
2 Ives, A.R. (1996) Evolution of insect resistance to Bt-transformed plants. *Science* 273, 1412–1413
3 McGaughey, W.H. *et al.* (1998) Bt resistance management. *Nat. Biotechnol.* 16, 144–146
4 Bowen, D. *et al.* (1998) Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* 280, 2129–2132
5 Guo, L. *et al.* (1999) *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins. *J. Biol. Chem.* 274, 9836–9842
6 Forst, S. *et al.* (1997) *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annu. Rev. Microbiol.* 51, 47–72
7 Forst, S. and Nealson, K. (1996) Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. *Microbiol. Rev.* 60, 21–43
8 Bowen, D.J. and Ensign, J.C. (1998) Purification and characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. *Appl. Environ. Microbiol.* 64, 3029–3035
9 Libby, S.J. *et al.* (1997) The *spv* genes on the *Salmonella dublin* virulence plasmid are required for severe enteritis and systemic infection in the natural host. *Infect. Immun.* 65, 1786–1792
10 Otto, H. *et al.* (2000) The *spvB* gene-product of the *Salmonella enterica* virulence plasmid is a mono(ADP-ribosyl)transferase. *Mol. Microbiol.* 37, 1106–1115
11 Crennell, S.J. *et al.* (2000) The predicted structure of photopexin from *Photorhabdus* shows the first haemopexin-like motif in prokaryotes. *FEMS Microbiol. Lett.* 191, 139–144
12 Riley, M.A. and Gordon, D.M. (1999) The ecological role of bacteriocins in bacterial competition. *Trends Microbiol.* 7, 129–133
13 ffrench-Constant, R.H. *et al.* (2000) A genomic sample sequence of the entomopathogenic bacterium *Photorhabdus luminescens* W14: potential implications for virulence. *Appl. Environ. Microbiol.* 66, 3310–3329
14 Jin, S. *et al.* (1996) Regulation of the *Serratia marcescens* extracellular nuclease: positive control by a homolog of P2 Ogr encoded by a cryptic prophage. *J. Mol. Biol.* 256, 264–278
15 Hurst, M.R. *et al.* (2000) Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. *J. Bacteriol.* 182, 5127–5138
16 Wang, Y.D. *et al.* (1998) Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. *J. Bacteriol.* 180, 4102–4110
17 Blackburn, M. *et al.* (1998) A novel insecticidal toxin from *Photorhabdus luminescens*: histopathological effects of toxin complex A (Tca) on the midgut of *Manduca sexta*. *Appl. Environ. Microbiol.* 64, 3036–3041
18 Relman, D.A. *et al.* (1989) Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. *Proc. Natl. Acad. Sci. U. S. A.* 86, 2637–2641
19 Leininger, E. *et al.* (1991) Pertactin, an Arg–Gly–Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 88, 345–349
20 Perry, R.D. and Fetherston, J.D. (1997) *Yersinia pestis*–etiologic agent of plague. *Clin. Microbiol. Rev.* 10, 35–66
21 Hinnebusch, B.J. *et al.* (1996) Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. *Science* 273, 367–370